CHANGES IN FUNCTIONAL PARAMETERS OF CRYOPRESERVED BOAR SPERMATOZOA CAPACITATED IN VITRO

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Abstract

The boar spermatozoa are extremely susceptible to freezing-thawing process and some damages can occur after their cryopreservation. The good functional state of cryopreserved and capacitated spermatozoa is crucial for standard in vitro fertilization and porcine embryo production. The aim of this study was to assess functional status of spermatozoa after cryopreservation, separation and capacitation to characterize the influence of these procedures on spermatozoa functional parameters. The ejaculates of 7 boars were frozen-thawed, motile spermatozoa were separated on Percoll-gradient and capacitated in Tris-buffered medium with 1mM caffeine at 39 ^{o}C in atmosphere of 5 % CO_2 for 3 hours. Motility, viability and chromatine integrity of spermatozoa were evaluated by phase contrast, Annexin V-FICT apoptosis detection kit and Sperm chromatin structure assay (SCSA) respectively. Data were analysed for high cryotolerant- (HCT-) boars (n = 4) and low cryotolerant- (LCT-) boars (n = 3) that were classified preliminary according to motility, viability and acrosome integrity of spermatozoa after semen thawing (Martečíková et al., 2009). There were no differences in motility, viability or chromatin integrity between spermatozoa of both boar groups before semen freezing but, the significantly ($P < 0.05$) higher motility and viability rates were found for spermatozoa of HCT- boars compared with those of LCT-boars after thawing. In HCT-boars, mean proportions of motile and viable spermatozoa decreased gradually during cryopreservation, separation and capacitation. The decrease in motility and viability of spermatozoa after thawing (from 78.8 ± 2.1 to 46.3 ± 7.8 % and from 85.3 ± 2.1 to 43.0 ± 7.3 % respectively) and capacitation (from 51.8 ± 5.5 to 36.8 ± 7.4 % and from 35.0 ± 3.7 to 29.9 ± 4.6 % respectively) was significant ($P < 0.05$). In LCT-boars, mean proportions of motile and viable spermatozoa decreased after thawing, (from 81.7 ± 5.7 to 16.7 ± 6.8 % and from 82.9 ± 2.1 to 39.1 ± 2.9 %), increased after separation (to 50.0 ± 6.3 % and 51.8 ± 5.5 %) and decreased again after capacitation (to 44.2 ± 3.7 % and 37.4 ± 9.0 % respectively). The all differences were significant ($P < 0.05$).

In both boar groups mean percentages of spermatozoa with intact DNA did not changed during the whole evaluated period. In conclusion, the cryotolerance of spermatozoa in boars cannot be predicted before cryopreservation of their semen. Separation of motile spermatozoa on a Percoll- gradient was more effective for the low- cryotolerant compared with the high-cryotolerant boars. The DNA of spermatozoa was highly stabile during cryopreservation, separation and capacitation by caffeine.

Key Words: Boar spermatozoa, Cryopreservation, Assessment, Annexin-V, SCSA

Cryopreservation of boar spermatozoa offers considerable advantages for the further development in pig reproductive industry. The frozen-thawed boar semen is still not used in pig reproduction usually, because of lower farrowing rate and litter size obtained after insemination of sows with cooled semen compared with that cryopreserved. It is widely known, that the boar spermatozoa are extremely susceptible to low temperatures and some damages can occur after their cooling or freezing and reduce their fertilizing capacity (Holt, 2000; Watson, 2000). The good functional status of frozen-thawed and capacitated spermatozoa is also crucial for effective oocyte fertilization and in vitro embryo production in pigs. Percoll is widely used for the selection of motile mammalian spermatozoa after cryopreservation and it was also successfully used in boars (Suzuki and Nagai, 2003). Capacitation of boar spermatozoa needs usually to be stimulated in porcine IVF systems. The most often agent, used for boar sperm capacitation is caffeine. But there are little knowledge about performance of boar spermatozoa after separation and caffeine treatment.

The evaluation of cryopreservated boar semen requires a wide variety of tests because a lot of spermatozoa functions are impaired during the freeze-thaw process. One of good indicators of spermatozoa functional status is assessment of membrane integrity. Disturbed membrane functions, consists in lose of asymmetry of the membrane phospholipids, occurs during the early phases of spermatozoa damage. This sign of early cell death could be monitored by *Annexin-V calcium-dependent*, phospholipid-*binding,* protein. It´s detection was described to be more sensitive than propidium iodide staining in cryopreservated human spermatozoa (Glander et al., 1999) and recently it was successfully applied on

evaluation of frozen-thawed boar spermatozoa membrane integrity (Pena et al., 2003). Another precondition of good spermatozoa functional status is integrity of DNA. A variety of methods have been developed for detecting different changes in the chromatine structure integrity (Choan et al., 2006). Sperm chromatine structure assay (SCSA) effective and sensible measures increased susceptibility of DNA to acid denaturation in situ using flow cytometry and it has been widely used for spermatozoa chromatine assessment (Evenson and Wixon, 2006).

In our preliminary study we modified the medium for cooling of boar semen before cryopreservation using trehalose. We evaluated basic functional parameters, motility, viability and acrosome integrity after cryopreservation and capacitation. This study was designed to assess the other important parameters of viability of spermatozoa, such as plasma membrane and chromatin integrity. The aim of the work was to characterize changes that could be developed in spermatozoa after their thawing, separation and capacitation with caffeine.

Material and Methods

Semen cryopreservation

The sperm rich fractions were collected from ejaculates of 2-5 year old boars (n= 7) of Large White and Pietrain breeds with proven fertility. The semen with at least 70% motility and 90% acrosome integrity of spermatozoa was equilibrated in cooling medium described earlier by Martecikova et al., 2008 and frozen by an adjusted procedure described by Westendorf et al. (1975) and subsequently modified by Cavarajal et al. (2004). After the equilibration in cooling extender (11 % egg yolk (v/v), 0.12 M lactose and 0.11 M trehalose) from 17 to 5°C, semen was resuspended in freezing extender (0,22 M lactose, 22.8% egg yolk (v/v), 1.3% (v/v), Equex STM, Minitübe, Tiefenbach, Germany and 7.5 % glycerol (v/v) to final concentration of 0.5×10^9 of spermatozoa/ml and frozen in 0.5 mL straws in liquid nitrogen vapour. The semen thawing was carried out by holding straws at room temperature for 15 s and plunging them into water bath at 37°C for 30 s.

Motile spermatozoa separation

After semen thawing, motile spermatozoa were separated on a 40 vs. 60% Percoll gradient by centrifugation at 520 g for 4 minutes. The pellet was removed and washed in BTS medium (Minitűbe, Tiefenbach, Germany).

Spermatozoa capacitation

The spermatozoa were resuspended (10×10^6 spermatozoa/ml) in modified Tris buffered medium (113.1mM NaCl, 3mM KCl, 10 mM, 10 mM $CaCl_2\times H_2O$, 20mM Tris, 11 mM glucose, 5mM sodium pyruvate and 0.4% BSA) with 1 mM or without caffeine as a control. Spermatozoa were capacitated in a humidified atmosphere of 5% CO_2 at 39°C for 3 hours.

Spermatozoa assessment

Motility, membrane and chromatin integrity of spermatozoa were assessed before freezing, thirty minutes after thawing, after separation and capacitation. Motility was evaluated subjectively using a phase contrast microscope. For spermatozoa membrane integrity ssessment the Annexin V-FITC apoptosis detection kit (Sigma chemicals, Prague, Czech Republic) was used. Three spermatozoa populations were distinguished: live spermatozoa (annexin-/propidium iodide-), annexin positive spermatozoa (annexin+/propidium iodide- or annexin+/propidium iodide+) and dead spermatozoa (annexin-/propidium iodide+).

The spermatozoa DNA integrity was evaluated by Sperm chromatin structure assay (SCSA, Evenson et al., 2002). Spermatozoa showing non-detectable DNA fragmentation were considered as DNA intact.

Statistical analysis

Results were analysed for high cryotolerant- (HCT-) boars (n = 4) and low cryotolerant- (LCT-) boars (n = 3) that were classified preliminary according to motility, viability and acrosome integrity of spermatozoa after semen thawing (Martečíková et al., 2009). Data were expressed as mean ± S.D. values and analysed by Student's t-test.

Results

There were no significant differences in motility, viability or DNA integrity between spermatozoa of LCT- and HCT- boars before semen freezing (Figure 1).On the other side, the significantly ($P < 0.05$) higher motility and viability rates were detected for spermatozoa of HCT- boars compared with those of LCT- boars after thawing (Figure 2). No differences in DNA integrity of spermatozoa between both groups boars either before or after semen thawing were found.

Motility, viability and DNA integrity of spermatozoa for the high cryotolerant- and low cryotolerant- boars are shown in the Table 1 and Table 2.

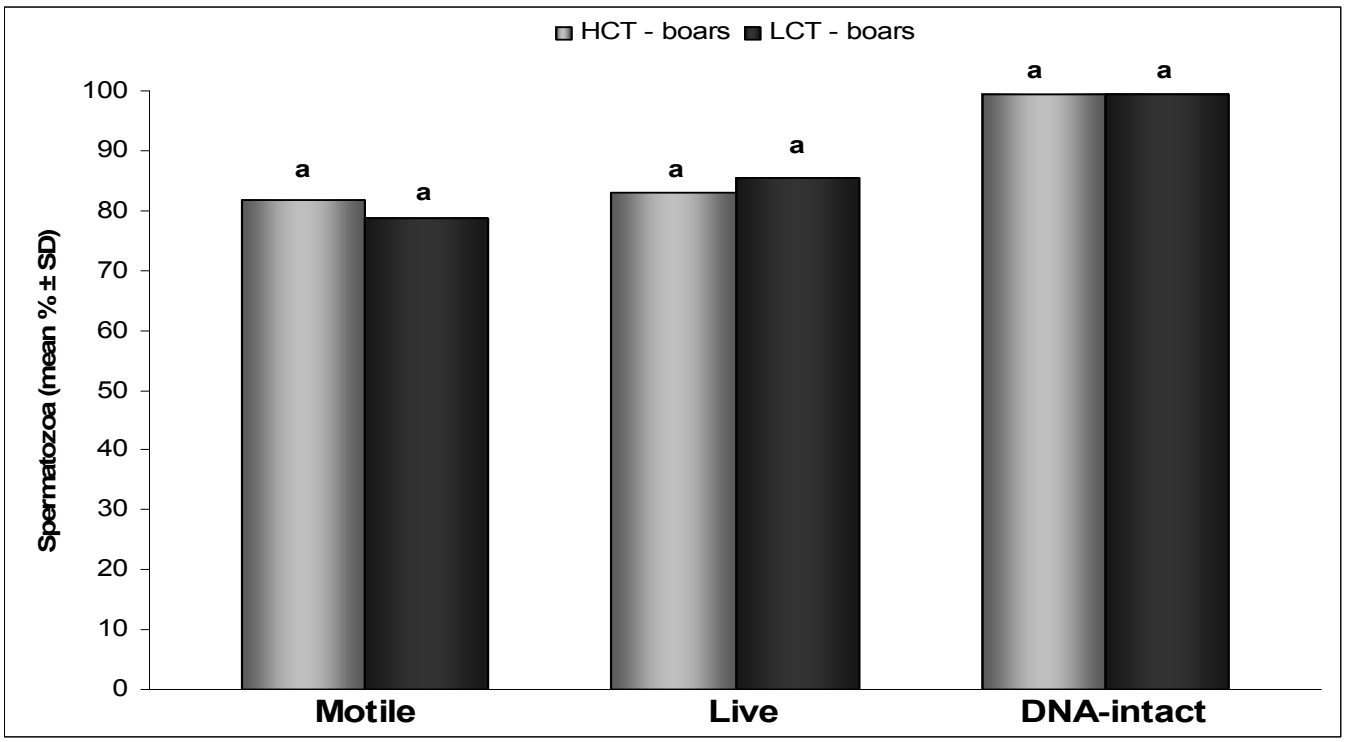
In HCT- boars, the mean proportions of motile and viable spermatozoa significantly ($P <0.05$) decreased after cryopreservation. Motility increased but viability decreased significantly ($P < 0.05$) after spermatozoa separation. Both motility and viability did also significantly ($P < 0.05$) decrease after capacitation (Table 1).

In LCT-boars, mean proportions of motile and viable spermatozoa decreased after thawing, increased after separation and decreased again after capacitation. The all differences were significant ($P < 0.05$, Table 2).

In both boar groups the mean proportions of annexin positive spermatozoa were very low before cryopreservation but significant ($P < 0.05$) increase in mean proportions of annexin positive spermatozoa was seen after thawing. In both boar groups the mean percentage of annexin positive spermatozoa increased even more after separation but it remained similar after capacitation.

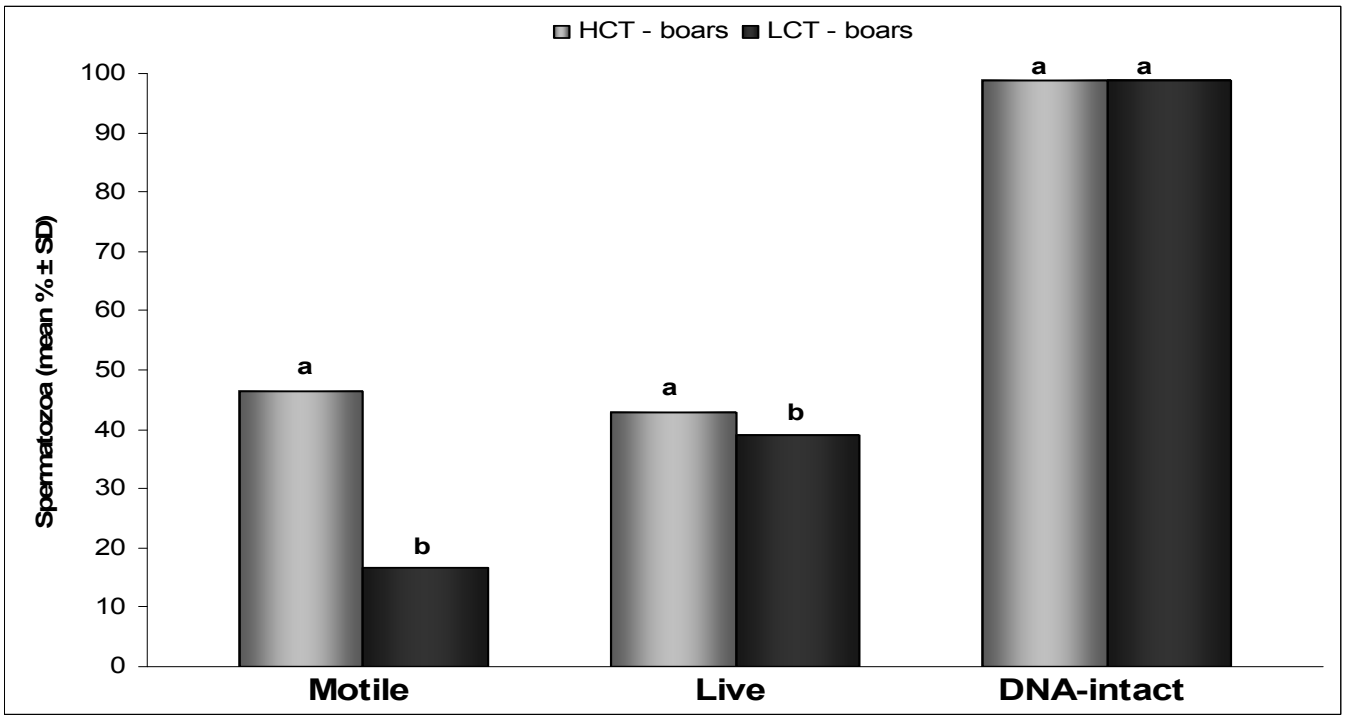
In both boar groups mean percentage of spermatozoa with intact DNA did not changed during the whole evaluated period.

Figure 1. Comparison of spermatozoa of high cryotolerant-boars and low-cryotolerant boars before freezing



Values with different superscripts values within the same evaluated parameter are significantly different for the same group at least at $P < 0.05$

Figure 2. Comparison of spermatozoa of high cryotolerant-boars and low-cryotolerant boars after thawing



Values with different superscripts values within the same evaluated parameter are significantly different for the same group at least at $P < 0.05$

Table 1. Characteristic of spermatozoa of HCT - boars after thawing, separation and caffeine capacitation

	Spermatozoa (mean% ± S.D.)				
	before freezing	after thawing	after separation	after caffeine treatment and capacitation	
				mM	3h
Motile	78.8 ± 2.1^{a}	46.3 ± 7.8^{b}	51.8 ± 5.5^{b}	1	36.8 ± 7.4^{c1}
				0	32.5 ± 5.8^{c1}
Live	85.3 ±1.2^{a}	43.0 ± 7.3^{b}	35.0 ± 3.7^{c}	1	29.9 ± 4.6^{d1}
				0	32.8 ± 2.6^{c1}
Annexin positive	5.1 ± 1.1^{a}	26.4 ± 14.0^{b}	52.2 ± 1.3^{c}	1	48.6 ± 4.9^{c1}
				0	48.0 ± 4.1^{c1}
Dead	9.6 ± 2.8^{a}	31.0 ± 11.1^{b}	12.8 ± 2.8^{c}	1	21.7 ± 5.6^{d1}
				0	21.9 ± 2.4^{d2}
DNA-intact	99.5 ± 0.3^{a}	98.8 ± 0.3^{a}	98.3 ± 0.6^{a}	1	98.0 ± 1.0^{a1}
				0	97.9 ± 0.1^{a1}

Values with different superscripts in the row (a-b, a-c, b-c) or column (1–2) are significantly different for the same group at least at$P < 0.05$

Table 2. Characteristic of spermatozoa of LCT - boars after thawing, separation and caffeine capacitation

	Spermatozoa (mean% ± S.D.)				
	before freezing	after thawing	after separation	after caffeine treatment and capacitation	
				mM	3h
Motile	81.7 ± 5.7^{a}	16.7 ± 6.8^{b}	50.0 ± 6.3b^{c}	1	44.2 ± 3.7^{d1}
				0	41.7 ± 5.2^{d1}
Live	82.9 ± 2.1^{a}	39.1 ± 2.9^{b}	51.8 ± 5.5^{c}	1	37.4 ± 9.0^{b,d1}
				0	42.2 ± 9.0^{b,d1}
Annexin positive	2.1 ± 1.3^{a}	28.1 ± 14.5^{b}	39 ± 4.1^{b}	1	42.2 ± 12.8^{b1}
				0	35.9 ± 8.5^{b1}
Dead	14.9 ± 3.8^{a}	32.1 ± 21.5^{b}	9.3 ± 2.6^{c}	1	20.4 ± 4.9^{b1}
				0	21.9 ± 2.4^{b1}
DNA-intact	99.4 ± 0.3^{a}	98.7 ± 0.7^{a}	98.9 ± 0.8^{a}	1	99.0 ± 0.6^{a1}
				0	98.8 ± 0.9^{a1}

Values with different superscripts in the row (a-b, a-c, b-c) or column (1–2) are significantly different for the same group at least at $P < 0.05$

Discusion

In our experiment, we studied the changes in functional status of cryopreserved boar spermatozoa prepared by the same method as those for in vitro fertilization. It is widely kown, that spermatozoa of the highest quality are required for succesfull oocyte fertilization (Gil et al., 2008).

In order to study a relationship between functional state of spermatozoa before and after semen freezing we categorized experimental boars into two groups according to cryotolerance of their spermatozoa. No differences in any of evaluated sperm parameters were found between high cryotolerant- and low cryotolerant- boars before semen freezing.

Different separation methods are widely used to get motile spermatozoa with normal morphology. Suzuki and Nagai (2003) described different influence of Percoll gradient separation on spermatozoa of individual boars. I our study, the influence of separation on spermatozoa was differed in boar groups. Separation of spermatozoa on Percoll gradient was more effective for LCT- compared with HCT- boars. It would be interesting to modify the separation procedure in terms of Percolls concentration or centrifugation conditions to enhance the efectivity of sperm separation in HCT- boars.

The exposure of Annexin V-binding sites in spermatozoa is related to loss of membrane phospholipids asymmetry. The AnnexinV-assay distinquishes four sperm populations usually: viable cells (annexin-/propidium iodide-), early apoptotic cells (annexin +/ propidium iodide -), late apoptotic (dead) cells (annexin +/ propidium iodide +) and dead non apoptotic cells (annexin -/ propidium iodide +). It is possible to speak about apoptosis of spermatozoa in the case of fresh semen. But we suppose that the mechanismus of annexin V-binding sites exposure in cryopreservated spermatozoa is another and it is probably related to spermatozoa membrane injury during cryopreservation. The cryoinjury influences the sperm membrane quality (Johnson et al., 2000). It was seen on increase of proportion of annexin positive spermatozoa which occured firstly after thawing and secondly after separation in our experiment. This was evident mainly in spermatozoa of HCT- boars, in which separation resulted in decrease of percentages of live, membrane intact but also dead spermatozoa. That is reason why we decided to join the two groups (annexin+/ propidium iodide- and annexin+/propidium iodide+) into annexin positive group.

The good DNA integrity in spermatozoa is required for successful oocyte fertilization and the relationship between sperm DNA integrity and sperm fertility was described (Bungum, et al., 2007). The SCSA has been used to assess sperm chromatin integrity in men, bulls and boars (Rybar et al., 2004; Reckova et al., 2008; Smit at al, 2007). In our study we confirmed the widely accepted opinion, that the cryopreservation has no effect on sperm DNA fragmentation. This was evident when we found the same DNA stability after cryopreservation in HCT- and LCT- boars. Further processing of spermatozoa, including separation and capacitation, had no effect on DNA fragmentation in our experiments.

Conclusion

It can be concluded that cryotolerance of spermatozoa of individual boars could not be predicted before cryopreservation. Separation of motile spermatozoa on Percoll-gradient was more effective for the low cryotolerant- compared with the high cryotolerant- boars. The DNA integrity of spermatozoa was highly stable during cryopreservation, separation and capacitation. The Annexin V-binding assay was the most sensitive method for assessment of sperm quality in comparison with other methods. The caffeine treatment was suitable for boar spermatozoa capacitation because it did not negatively influence their functional status.

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